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CONCERNING A FILING UNDER 35 U.S.C. 371

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INTERNATIONAL APPLICATION NO.

PCT/US98/26788

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PRIORITY DATE CLAIMED

17 December 1997 (17.12.97)

TITLE OF INVENTION

METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE PHENOTYPE OF SOFT TISSUE CELLS

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Postcard

Check

Express Mail Label No. EL390998886US

Date of Deposit: 16 June 2000 (16.06.00)

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ATTORNEY'S DOCKET NUMBER

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21. The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**\$840.00**

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	19 - 20 =	0	x \$18.00
Independent claims	2 - 3 =	0	x \$78.00

\$0.00**\$0.00**Multiple Dependent Claims (check if applicable). ☐**\$0.00****TOTAL OF ABOVE CALCULATIONS =****\$970.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). ☐

\$0.00**SUBTOTAL =****\$970.00**

Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00**TOTAL NATIONAL FEE =****\$970.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐

\$0.00**TOTAL FEES ENCLOSED =****\$970.00**

Amount to be:
refunded \$
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☒ A check in the amount of **\$970.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0311** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Limited Recognition

REGISTRATION NUMBER

16 June 2000

DATE

**METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE
PHENOTYPE OF SOFT TISSUE CELLS**

This application claims the benefit of U.S. Provisional Application No. 60/069,931, filed December 17, 1997, and U.S. Provisional Application No. 60/110,498, filed December 1, 1998.

Field of the Invention

The present invention relates generally to methods for maintaining or restoring tissue-appropriate phenotype in soft tissue cells. More particularly, the invention relates to methods for maintaining or restoring tissue-appropriate phenotype of diseased, damaged, or aged soft tissue by manipulating a regulatory pathway leading to phenotype-specific protein expression.

Background of the Invention

Numerous factors are known to influence cellular growth, differentiation, and maintenance. One of the most important groups of growth and differentiation factors are members of the TGF- β family, particularly the morphogens, including members of the family of bone morphogenic proteins, first identified by their ability to induce endochondral bone morphogenesis. However, they have now been recognized as one of the group of general growth and differentiation factors that are capable of sustaining growth and differentiation in tissue generally. In addition, morphogens have been implicated in cellular apoptosis.

As used herein, the terms "morphogen," "bone morphogen," "bone morphogenic protein," "BMP," "morphogenic protein" and "morphogenetic protein" all embrace the class of proteins typified by human osteogenic protein 1 (hOP-1). Nucleotide and amino acid sequences for hOP-1 are shown in SEQ ID NO: 7. For ease of description, hOP-1 is a representative morphogen. It is appreciated that OP-1 is merely representative of the TGF- β subclass of true tissue morphogens, and is not intended to limit the description. Preferred morphogens are those that share at least 60% amino acid sequence identity or preferably at least 70% amino acid sequence homology with the C-terminal seven cysteine domain of hOP-1. Other known and useful morphogens include, but are not limited to, the mammalian osteogenic protein-1 (OP-1, also known as BMP-7, and the *Drosophila* homolog 60A), osteogenic protein-2 (OP-2, also known as BMP-8), osteogenic protein-3 (OP-3), BMP-2 (also known as BMP-2A or CBMP-2A,

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and the *Drosophila* homolog DPP), BMP-3, BMP-4 (also known as BMP-2B or CBMP-2B), BMP-5, BMP-6 and its murine homolog Vgr-1, BMP-9, BMP-10, BMP-11, BMP-12, GDF3 (also known as Vgr2), GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, BMP-13, BMP-14, BMP-15, GDF-5 (also known as CDMP-1 or MP52), GDF-6 (also known as CDMP-2), GDF-7 (also known as CDMP-3), the *Xenopus* homolog Vgl and NODAL, UNIVIN, SCREW, ADMP, and NEURAL, and morphogenically-active amino acid variants (such as conservative substitution variants) of any thereof. Typically, such morphogens share functional features, such as the ability to stimulate endochondral bone formation in an *in vivo* bone assay, or the ability to stimulate N-CAM or L1 isoform production in an NG108-15 neuronal cell culture. See U.S. 4,968,590; Sampath et al., *Proc. Natl. Acad. Sci. USA* 80: 6591-6595 (1983), incorporated by reference herein. Other functional assays for morphogen activity, useful in identifying morphogens are known in the art.

Morphogens include secretory peptides sharing common structural features. Typically, the mature form of the protein is processed from a precursor "pro-form." The mature form is a dimer containing a carboxy terminal active domain having approximately 97-106 amino acids, containing a conserved pattern of cysteines. The active form is either a disulfide-bonded homodimer or a heterodimer. See, e.g., Massague, *Annu. Rev. Cell Biol.* 6:597 (1990); Sampath et al., *J. Biol. Chem.* 265:13198 (1990). While the morphogens have significant homologies and similarities in structure, variants within the morphogenic protein genes may have specific roles in specific tissue involving, for example, stimulation of progenitor cell multiplication, tissue specific or tissue preferred phenotype maintenance, and/or stimulation or modulation of the rate of differentiation, growth or replication of tissue cells characterized by high turnover.

The morphogenic activities of the TGF- β superfamily of proteins allow them to initiate and maintain the developmental cascade of tissue morphogenesis in an appropriate, morphogenically-permissive environment, stimulating stem cells to proliferate and differentiate in a tissue-specific manner, and inducing the progression of events that culminate in new tissue formation. Specifically, morphogens are capable of at least the following biological functions in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated

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cells; and supporting the growth and maintenance of differentiated cells, including the "redifferentiation" of transformed cells.

These morphogenic activities also allow the proteins to stimulate the "redifferentiation" of committed cells previously induced to alter their phenotype due to disease, damage, or age.

5 Morphogens are useful in the replacement of diseased, damaged, or aged tissue, particularly when the damaged tissue interferes with normal tissue or organ function. For example, elevated morphogen expression induces repair of damaged lung tissue resulting from emphysema; damaged kidney cells; cirrhotic liver cells; damaged heart or blood vessel; damaged stomach tissue resulting from ulcers or their repair; damaged neural tissue (*e.g.*, resulting from stroke) or neuropathies such as Alzheimer's disease, Parkinson's disease, Huntington's chorea, and multiple sclerosis; damaged skeletal or orthopedic tissues; or damaged dentin and periodontal tissues as may result from disease or mechanical damage or injury. Furthermore, morphogens are useful in treating symptoms resulting from diseased, damaged, or aged soft tissue cells, such as pain, including neuropathy pain.

10 Morphogens act to induce an intracellular cascade that results in expression of phenotype-specific gene products. Such gene products include proteins necessary or sufficient to maintain, enhance, or restore phenotype, including structural proteins, enzymes, and the like. Generally, a morphogen acts as a ligand for specific Type I and/or Type II transmembrane receptors, each receptor typically being associated with a serine/threonine kinase. In a common scenario, after
15 ligand binding, a Type II receptor phosphorylates an adjacent Type I receptor. The activated Type I receptor recognizes specific members of the Smad protein family, phosphorylating them at least at the carboxy-terminal serine residue. Eight different Smad proteins have been identified in mammals. These are classified into three subgroups, including pathway-restricted Smads (R-Smads), common-mediator Smads (co-Smads), and inhibitory Smads (anti-Smads).
20 R-Smads are directly activated by Type I receptors, form complexes with co-Smads, and translocate into the nucleus. The Smad heteromers directly bind to DNA, and also associate with other DNA binding proteins, and thus regulate the transcription of target genes. Smad1, 5, and 8 are activated by BMP receptors, and Smad2 and 3 are activated by TGF- β and activin receptors. Smad4 functions as a co-Smad. Smad6 and Smad7 are distantly related in terms of structure
25 with other Smads, and serve as anti-Smads.
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The Smad1, Smad2, Smad3 and Smad5 proteins consist of conserved amino- and carboxy-terminal domains linked by a region that is more divergent among the Smads. The carboxy-terminal domain has an effector function. The amino-terminal domain interacts physically with the carboxy-terminal domain, inhibiting its effector activity, and contributes to DNA binding. Receptor-mediated phosphorylation of the serine residues at the end of the carboxy-terminal domain relieves the carboxy-terminal domain from the inhibitory action of the amino-terminal domain. Phosphorylated Smad molecules form a heteromeric complex with at least one other specific Smad family molecule. The resulting Smad complex then translocates into and accumulates in the cell nucleus. There, the heteromeric Smad complexes regulate transcriptional responses either alone or by specific interaction with a DNA-binding protein, such as forkhead activin signal transducer-1 (FAST1).

With particular reference to the OP-1 or BMP-2 activated pathway, as shown in Figure 2, morphogens are ligands for the Type I and Type II receptors. The Type II receptor comprises a constitutively-active kinase, which transphosphorylates a Type I receptor upon ligand binding. Following phosphorylation of the Type I receptor by the Type II receptor, the Type I receptor specifically phosphorylates Smad1 homodimers. The Type I receptor also specifically phosphorylates Smad5 homodimers. The homodimers then separate to form, in association with a phosphorylated Smad4 molecule, a phosphorylated heteromeric complex comprising at least a Smad1 and a Smad4. A phosphorylated Smad1/Smad5/Smad4 heterotrimer may alternatively be formed. The heteromeric complex then translocates into the nucleus, and accumulates therein. In the nucleus, the Smad complex binds operative DNA, either alone or in association with a specific DNA binding protein (the X-protein in Figure 2), to initiate DNA transcription. The "X-protein" acts as a DNA-binding protein, binding the Smad heteromeric complex to the DNA. The pathway leading to endogenous morphogen expression is similar to the one described above, with the Smad heteromeric complex inducing transcription of the morphogen-encoding gene. Other intracellular pathways are induced by morphogens, and may be affected in the manner described herein.

Diseased, damaged, or aged soft tissue cells are characterized in part by a decrease in endogenous expression of morphogenic protein, and OP-1 in particular. This decrease in endogenous expression of morphogenic protein causes the cells to dedifferentiate, displaying

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tissue-inappropriate phenotype which, at the extreme, results in cell death. For example, cells in the substantia nigra of the brain progressively become dysfunctional in patients with Alzheimer's disease. Similarly, liver cells may lose their phenotype (*i.e.*, become cirrhotic) due to alcohol abuse or other causes. Accordingly, there is a need in the art for methods to stimulate diseased, 5 damaged, or aged cells to maintain or to restore tissue-appropriate phenotype.

Summary of the Invention

It has now been recognized that preservation and maintenance of cellular phenotype is accomplished by activation of pathways that normally are modulated by growth and differentiation factors. Moreover, inhibition of those pathways is now recognized as an 10 additional means for preserving or inducing appropriate phenotype.

Normal phenotype is controlled not only by developmental cues, but also by various endogenous growth factors. However, disease, injury or aging may affect one or more aspects of cellular function, including the ability of growth and differentiation factors to modulate gene expression leading to normal cellular phenotype. For example, chronic degenerative illness may result not only in biochemical dysfunction, but in the inability of affected tissue to replace lost cells. 15

The present invention comprises activating and controlling phenotypic effects through action at various intracellular pathways. In so doing, morphogen-activated pathways are used as an example of the ways in which tissue growth and differentiation can be modulated. However, 20 methods disclosed herein are useful in the restoration and/or maintenance of phenotype through action at any intracellular pathway that is normally modulated by any growth and differentiation factor.

Accordingly, the present invention provides methods for maintaining or restoring tissue-appropriate phenotype in a soft tissue cell. According to methods of the invention, tissue-appropriate phenotype is maintained or restored by increasing endogenous expression of a 25 phenotype-specific protein. In a preferred embodiment, endogenous phenotype-specific protein expression is increased by manipulating an intracellular regulatory pathway that modulates expression of a gene encoding such a protein. Thus, methods of the invention comprise administering a composition that interacts with at least a portion of an intracellular pathway by

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which growth and maintenance factors, such as morphogens, cause expression of a phenotype-specific protein (*e.g.*, a protein associated with preservation, restoration, or enhancement of phenotype). Such methods also comprise exposing cells to a composition that interacts with a pathway by which endogenous growth and maintenance factor production is stimulated, thereby to stimulate an increase in growth and maintenance factor expression by the cell. In a preferred embodiment, methods of the invention comprise detecting the component or components of cellular biology that are lacking in a given cell or cell population, and then targeting activation or inhibition of a pathway, the result of which is compensation or restoration of the lacking component(s).

Methods of the invention involve exogenous stimulation of one or more components of a pathway that normally leads to the expression of a phenotype-specific gene product. Expression of a particular phenotype-specific gene may be the direct and immediate result of the modulation of a particular pathway or may be the result of any one or more biochemical effects flowing from such modulation. For example, modulation a particular pathway may result in an increase of endogenous expression of OP-1, which in turn may activate a different regulatory pathway that ultimately results in increased expression of a phenotype-specific gene product. A prototypical pathway is shown in Figure 2. As shown in the Figure, receptor binding activates an intracellular kinase, causing phosphorylation of intracellular messenger molecules called Smads. The Smads have been characterized, and are known in the art. *See, e.g.*, Baker et al., *Curr. Op. Genet. Devel.*, 7: 467-473 (1997), incorporated by reference herein. Upon phosphorylation, various Smad subtypes form complexes which then translocate into the nucleus. Once in the nucleus, Smad complexes, either on their own or in association with a transcription activator, modulate, either directly or indirectly, expression of specific gene product that is characteristic of the stimulated cell. One such gene product is a morphogen itself. Thus, the pathway may play a role in positive feedback on morphogen expression, thus affecting another pathway resulting in phenotype-specific gene expression.

Methods of the invention may also be used to inhibit the effects of cellular components that diminish normal cellular phenotype. Whether any specific cellular component enhances or diminishes cellular phenotype depends upon the local environment, the developmental state, and the disease/injury status of the cell. For example, during wound healing transforming growth

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factor- β (TGF- β) promotes formation of scar tissue via fibrosis. In wound healing studies, morphogens have been shown to counteract this effect. It has also been shown that two particular Smad proteins, Smad6 and Smad7, inhibit TGF- β . Accordingly, in one embodiment, methods of the invention comprise activating Smad6/Smad7 to inhibit TGF- β at the site of wound healing. Additional activation of morphogen-induced intracellular pathways further promotes healing, and the presentation of normal cells at the wound site. The precise nature of regulation according to methods of the invention depends upon the environment in which the tissue exists, the age of the tissue, and the disease or injury state of the tissue. However, activation of morphogen-induced pathways as described herein results in restoration and maintenance of normal phenotype. Precise control over biochemical functions is achieved by targeting specific pathways that have been adversely affected by age, disease, and/or injury.

In another aspect, the invention provides methods for increasing the level of endogenous phenotype-specific protein comprising the step of introducing a small molecule that regulates some portion or portions of a cellular regulatory pathway, resulting in an effective increase in expression or activity of a phenotype-specific protein. This may result either from stimulating an increase in the endogenous expression of a phenotype-specific protein or a decrease in the expression or inhibitory activity of an inhibitor of normal (in the appropriate developmental and anatomical context) phenotype. For example, a small molecule may act at the Type I or Type II morphogen receptor; or at the serine/threonine kinase, or other kinase domains of those receptors. Another target of pathway activation is the Smad proteins, including the monomeric, dimeric (including heteromeric and homomeric complexes) or trimeric forms (including heteromeric and homomeric complexes). Alternately, activation of a transcription factor (for example, the X-protein shown in Figure 2) will lead to phenotype-specific expression. A small molecule may act to facilitate, mimic, or, if desired, prevent any one or several of the following: Type I and/or Type II receptor binding, phosphorylation of the Type I receptor, phosphorylation of the Smad molecules, Smad complex formation, Smad translocation into the nucleus, nuclear accumulation of the Smad complex, or transcription modulation of the Smad complex. Furthermore, a small molecule may act on Smads or Smad complexes to alter tertiary structure, thereby to facilitate or inhibit interaction of the Smad or Smad complex with a receptor kinase domain, other Smads, DNA binding proteins, or DNA itself.

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In a particularly-preferred embodiment, a small molecule is administered to a patient, wherein the small molecule facilitates formation of Smad complexes, particularly complexes comprising molecules of Smad1, Smad2, Smad3, Smad4, Smad5 and/or Smad8 in order to facilitate phenotype-specific gene expression. Also in a preferred embodiment, methods
5 comprise administering a composition that activates a serine/threonine kinase domain associated with a morphogen Type I or Type II receptor, thereby to activate the pathway involved in morphogen-induced gene expression. In another embodiment, methods of the invention comprise activating Smad4 association with Smad1, thereby to induce morphogen-responsive phenotypic gene expression. Methods of the invention may also facilitate Smad interaction with
10 specific nucleic acids, such as promoters of phenotype-specific gene expression (*i.e.*, expression of genes for a phenotypic protein; a protein associated with preservation, restoration, or enhancement of phenotype, including a protein which is critical for production of non-protein phenotypic markers, such as characteristic lipids or carbohydrates; a protein associated with performance of a phenotypic function or morphology; or a morphogen). Such interaction may
15 be, for example, in association with a transcription control factor that is capable of binding to a regulatory portion of a gene and, simultaneously, to one or more regulatory proteins such as a Smad complex (see Figure 2). As used herein, phenotype-specific gene expression or morphogen-induced gene expression refers to the expression of genes that are under morphogen control, or can be controlled by morphogens in a normal, healthy cell.

20 In another aspect of the invention, enhancement, preservation or restoration of phenotype may be achieved by providing a small molecule that acts as an agonist at the morphogen Type I or Type II receptor, thereby to stimulate activation of the pathway leading to phenotype-specific gene expression or to morphogen expression.

Methods of the invention also comprise the step of administering a composition
25 comprising a small molecule capable of decreasing inhibition of morphogen-induced phenotype-specific protein expression. Morphogen inhibition may be in the form of endogenous inhibitory compounds, such as leukemia inhibitory factor or cytokines, or may be in the form of exogenously applied inhibitors. Furthermore, Smad6 and/or Smad7 have inhibitory activity on the regulatory pathway of phenotype-specific protein expression. Accordingly, methods of the
30 invention also comprise affecting Smad6 and/or Smad 7 activity.

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The present invention further provides methods for treating a soft tissue disorder characterized by decreased levels of morphogen expression. Disorders characterized by decreased levels of morphogen expression resulting in dedifferentiation of soft tissue cells include lung damage caused by emphysema; cirrhotic kidney or liver tissues; damaged muscle tissue; damaged heart or blood vessel tissues, as may result from cardiomyopathies and/or atherothrombotic or cardioembolic strokes; damaged stomach or intestinal tissues resulting from ulceric perforations or their repair; and damaged neural tissue (including visual and auditory sensory tissue) as may result from physical or chemical injury, such as strokes, or neuropathies such as Alzheimer's disease, Parkinson's disease, Huntington's chorea, or multiple sclerosis, or neuropathic or other pain associated with any of the foregoing. Methods of the invention may comprise the step of administering a small molecule affecting a pathway leading to expression of phenotype-specific genes, or may comprise administering exogenous morphogenic protein, or an agonist thereof, including the monomer, dimer and/or soluble complex forms (comprising one or more morphogen pro domain noncovalently associated with a morphogen dimer), to the tissue locus having diseased, damaged, or aged soft tissue cells. The composition comprising the morphogenic protein may further comprise a matrix. Useful matrix materials include collagen, demineralized bone, hydroxyapatites, bioactive ceramics, calcium phosphate ceramics or mixtures comprising any one or more of the foregoing materials.

The present invention further provides methods for treating a soft tissue disorder characterized by decreased levels of morphogenic protein expression comprising the step of administering a composition comprising a morphogen and a small molecule capable of releasing inhibition of phenotype-specific protein expression.

In yet another aspect, the invention provides *in vivo* methods for increasing the level of endogenous expression of morphogenic protein or a phenotype-specific protein, comprising the step of administering naked DNA or mRNA encoding a morphogenic protein or a phenotype-specific protein directly to the locus of damaged, diseased or aged soft tissue cells. See U.S. Patent No. 5,580,859, the teachings of which are incorporated by reference herein. In another aspect, the invention provides *ex vivo* methods for increasing the level of endogenous expression of morphogenic protein or a phenotype-specific protein comprising introducing DNA encoding a morphogenic protein or a phenotype-specific protein into a soft tissue cell, and placing the

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transformed soft tissue cell at the tissue locus having damaged, diseased or aged soft tissue cells. Introduction of the DNA encoding a morphogenic protein or a phenotype-specific protein into a soft tissue cell may be accomplished by a variety of means and methods, including the use of plasmid DNA; viral vectors, including retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, SV40, polyoma virus, papilloma virus and picornavirus; DNA on the interface of, or encapsulated in liposomes or proteoliposomes; calcium phosphate, DEAE-Dextran; polybrene; polysine/DNA conjugates; or electroporation or microinjection.

Furthermore, the present invention provides methods for treating soft tissue disorders by affecting apoptosis by modulating a morphogen-activated regulatory pathway. Apoptosis is a distinctive form of cell death manifested by characteristic chromatin condensation and DNA fragmentation, resulting in the programmed death of cells as part of the normal cell cycle. However, apoptosis may also be induced by pathologic stimuli. In some instances, gene transcription and protein synthesis are required for the induction of apoptosis, and the process is regulated by a set of genes that are involved in normal cell growth and differentiation.

Apoptosis is responsible for numerous physiologic and pathologic events. For example, apoptosis is responsible for the programmed destruction of cells during embryogenesis (including implantation, organogenesis, developmental involution) and metamorphosis. Apoptosis is also responsible for hormone-dependent involution in the adult, such as endometrial cell breakdown during the menstrual cycle, ovarian follicular atresia in the menopause, and the regression of the lactating breast after weaning. Apoptosis also functions in cell deletion in proliferating cell populations, such as intestinal crypt epithelia. In addition, apoptosis is responsible for cell death in tumors, most frequently during regression but also in tumors with active cell growth. Moreover, apoptosis is responsible for the death of immune cells, both B and T lymphocytes after cytokine depletion, as well as deletion of autoreactive T cells in the developing thymus. Furthermore, apoptosis is responsible for pathologic atrophy of hormone-dependent tissues, such as prostatic atrophy after castration and loss of lymphocytes in the thymus after glucocorticoid administration. Apoptosis is also responsible for pathologic atrophy in parenchymal organs after duct obstruction cell death induced by cytotoxic T cells, such as in cellular immune rejection and graft-versus-host disease. In addition, apoptosis plays a role in cell injury in certain viral diseases, as for example in viral hepatitis, in which apoptotic cell

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fragments in the liver are known as Councilman bodies. Apoptosis is also responsible for cell death produced by a variety of injurious stimuli, including mild thermal injury, radiation, cytotoxic anticancer drugs, and possible hypoxia, that are capable of producing necrosis, but when given in low doses, induce apoptosis.

5 The pathways by which apoptosis is induced vary, depending on the stimulus and cell type. One important feature of apoptosis is its dependence in many (but not all) instances on gene activation and new protein synthesis. A number of genes can be induced by stimuli causing apoptosis, such as heat-shock proteins and proto-oncogenes. Apoptosis-specific genes that stimulate or inhibit cell death have been described. Certain genes involved in growth and in the causation of cancer (oncogenes and suppressor genes) play a regulatory role in the induction of apoptosis. These include the bcl-2 oncogene which inhibits apoptosis induced by hormones and cytokines and thus extends cell survival; the c-myc oncogene, whose protein produce can stimulate either apoptosis of cell growth; and p53 which normally stimulates apoptosis, but when mutated or absent, favors cell survival. However, in many models of apoptosis, new gene expression is not required and indeed inhibition of gene expression causes apoptosis. Accordingly, the methods of the invention also provide for promoting or inhibiting apoptosis in a soft tissue cell by modulating a morphogen-activated regulatory pathway.

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20 Methods of the invention are carried out in any tissue having diminished or lost phenotypic function as a result of disease, injury, or aging. Alternatively, methods of the invention are applicable in developing embryonic tissue.

 The preferred methods and examples that will now be described are illustrative only and are not intended to be limiting. Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

25 Figure 1 is a tabular presentation of the percent amino acid sequence identity and percent amino acid sequence homology ("similarity") that various members of the family of morphogenic proteins as defined herein share with hOP-1 in the C-terminal seven cysteine skeleton.

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For example, hepatocytes are large, polyhedral cells that have a variable cytoplasmic appearance depending on the nutritive status of the body. The nuclei of hepatocytes are large with peripherally dispersed chromatin and prominent nucleoli. The nuclei, however, vary greatly in size. This reflects the fact that more than half the normal complement of hepatocytes contain
5 twice the normal amount of chromosomal material, some contain four or even eight times the normal amount. Hepatocytes generally perform the phenotypic functions of storing glycogen, fat, and certain vitamins. Hepatocytes also transform nutritive substances in the diet into one another to deliver into the blood a needed nutrient. For example, hepatocytes can transform protein into carbohydrate, if needed. Furthermore, hepatocytes will transform and/or conjugate
10 certain products in order to detoxify them. In addition, hepatocytes regulate the concentration of certain substances, such as sugar, in the blood. Hepatocytes also express such phenotypic proteins as albumins, fibrinogens and globulins. Morphogens restore healthy phenotype to hepatocytes that have lost one or more structural or biochemical function characteristic of normal function. Once identified, the diminished phenotypic function is restored, in whole or in part, by
15 activation of pathways leading to phenotype-specific protein production. Typically, such pathways are under morphogen regulation.

Those practicing in the art will appreciate that phenotype-specific markers, functions and morphology for other cell types are well-known.

Small Molecule-Mediated Upregulation

20 The pathways that regulate gene expression are affected by a wide variety of developmental and environmental stimuli, thus allowing each cell type to express a unique and characteristic subset of its genes, and to adjust the expression of particular gene products as needed. The importance of expression control is underscored by the fact that targeted disruption of key regulatory molecules in mice often result in drastic phenotypic abnormalities, just as
25 inherited or acquired defects in the function of genetic regulatory mechanisms contribute broadly to human disease. Of interest in this regard is the usefulness of small molecules capable of controlling expression of phenotype-specific genes. Morphogen-activated regulatory pathways may be modulated by, for example, administering a small molecule capable of stimulating the expression of a phenotype-specific protein.

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A small molecule may be a morphogen analog that mimics activation of the regulatory pathway by a morphogen, such as OP-1. Small molecule morphogen analogs are identified, for example, as reported in co-pending patent application, U.S.S.N. 08/507,750, incorporated by reference herein. Any substance having such mimetic properties, regardless of the chemical or biochemical nature thereof, is useful as a morphogen analog as taught herein. The present morphogen analog may be a simple or complex substance produced by a living system or through chemical or biochemical synthetic techniques. It may be a substance that occurs in nature or a novel substance, *e.g.*, prepared according to principles of rational drug design. It may be a substance that structurally resembles a solvent-exposed morphogen surface epitope implicated in receptor interactions, a substance that otherwise stimulates a transmembrane morphogen receptor, or a cell-membrane permeant substance that interacts with any one or more intracellular aspects of the signal transduction pathway of a morphogen responsive cell. For example, a naturally-sourced OP-1 or morphogen analog may comprise a polypeptide, polynucleotide, carbohydrate, lipid, amino acid, nucleic acid, sugar, fatty acid, steroid, or a derivative of any one of the aforementioned compounds. It may be an intermediate or end product of metabolism of a eukaryotic or prokaryotic cell. Alternatively, the analog may be a biological response modifier or a toxin.

Without being limited, one type of morphogen analog useful in the methods of the present invention can be prepared through application of the principles of biosynthetic antibody binding site (BABS) technology as set forth in U.S. Patent Nos. 5,132,405, 5,091,513 and 5,258,498, the teachings of which are incorporated by reference herein. BABS analog constructs are prepared from antibodies, preferably produced by hybridoma cells, that bind specifically to a morphogen transmembrane receptor. Alternatively, BABS analysis is based upon anti-idiotypic antibodies specifically reactive with the antigen binding site of an antibody that blocks morphogen biological activity. Vukicevic et al., *Biochem. Biophys. Res. Comm.* 198: 693-700 (1994), teaches the preparation of OP-1 specific monoclonal antibodies. Skilled artisans will appreciate that such antibodies can be used as immunogens in the routine preparation of anti-idiotypic antibodies from which BABS analogs of the present invention can be prepared.

A structurally distinct class of morphogen analogs, again set forth herein for illustration and not for limitation, can be prepared through application of the principles of directed molecular

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evolution as set forth in Tuerk et al., *Science* 249:505-510 (1990), Famulok et al., *Angew. Chem. Intl. Ed. Engl.* 31:979-988 (1992) and Bock et al., *Nature* 355:564-556 (1992), the teachings of each of which are incorporated by reference herein. The directed molecular evolution process involves isolation of a nucleic acid molecule, typically an RNA, that binds with high affinity to a selected ligand such as a protein. Such a nucleic acid molecule is referred to in the art as an "aptamer." The desired aptamer is initially present in a random pool of nucleic acid molecules, and is isolated by performing several rounds of ligand-affinity based chromatography alternating with PCR-based amplification of ligand-binding nucleic acids. Bock et al., (1992), above, have demonstrated the preparations of aptamers, suitable for *in vivo* use in mammals, that specifically inhibit the blood clot promoting factor, thrombin.

Yet another structurally distinct class of morphogen analogs is prepared by selecting appropriate members of a random peptide library (Scott et al., (1990) *Science* 249:386-390), or a combinatorially synthesized random library of organic or inorganic compounds. Needels et al., *Proc. Natl. Acad. Sci. USA*, 90:10700-10704 (1993); Ohlmeyer et al., *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993). Skilled artisans appreciate that the foregoing and other related technologies, taken together with long-established principles of screening biologically-produced substances, offer a wide array of candidate compositions for screening for morphogen analog activity.

Thus, as used herein, a morphogen analog is a substance that mimics morphogen activation of the regulatory pathway of phenotype-specific gene expression inducing at least one "morphogen-mediated biological effect" in a cell or tissue. The effect can be any biological effect resulting from exposure to or contact with a morphogen, including but not limited to maintenance or restoration of tissue-appropriate phenotype. Morphogen-mediated biological effects include cellular and molecular responses to morphogen exposure, as described, for example, in co-pending patent application U.S.S.N. 08/260,675 and U.S. Pat. No. 5,656,593, the disclosures of which are incorporated by reference herein. Thus, it is appreciated that a morphogen-mediated biological effect is any biological effect resulting from exposure to or contact of morphogen-responsive cells or tissue with a morphogen, whether *in vitro* or *in vivo*. A morphogen-mediated biological effect of particular interest herein includes stimulation of the expression of one or more phenotype-specific genes, including stimulation of the binding of an

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intracellular substance to DNA expression regulation elements. Preferred morphogen-mediated biological effects include maintenance of a differentiated phenotype, or induction of redifferentiation, and/or stimulation of cellular proliferation and cellular differentiation.

In a highly-preferred embodiment, the small molecule is a compound that affects at least one intracellular pathway that normally is under morphogen regulation. Such small molecules preferably have the ability to enter the cell and target one or more intracellular pathway components in order to stimulate or inhibit its activity. For example, a small molecule that promotes Smad complex formation between Smad1, Smad4, and Smad5 will stimulate pathways leading to expression of genes encoding phenotype-specific proteins.

One way in which to identify a candidate small molecule is to assay for the ability of the candidate to modulate the effective systemic or local concentration of a morphogen. This may be done, for example, by incubating the candidate in a cell culture that produces the morphogen, and assaying the culture for a parameter indicative of a change in the production level of the morphogen according the methods of U.S. Pat. No. 5,741,641 and/or U.S. Pat. No. 5,650,276, the teachings of each of which are incorporated by reference herein. Alternatively, candidate compounds are screened for their ability to induce phenotype-specific protein production in a cell culture in which morphogen activity is not present. Examples of compositions which may be screened for their effect on the production of morphogens or other phenotype-specific proteins include but are not limited to chemicals, biological response modifiers (*e.g.*, lymphokines, cytokines, hormones, or vitamins), plant extracts, microbial broths and extracts medium conditioned by eukaryotic cells, body fluids, or tissue extracts. Useful candidate compositions then may be tested for *in vivo* efficacy in a suitable animal model. These compositions then may be used *in vivo* to upregulate morphogen-activated regulatory pathways of phenotype-specific protein expression.

A simple method of determining if a small molecule compositions has affected a change in the level of a phenotype-specific protein in cultured cells is provided in U. S. Pat. No. 5,741,641, the disclosure of which is incorporated by reference herein. The level of a target phenotype-specific protein in a cell resulting from exposure to a small molecule is measured. Alternatively, a change in the activity or amount of an intracellular pathway component is measured in response to application of a candidate small molecule. Candidates having the

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desired affect on protein production or pathway regulation are selected for use in methods of the invention. If, for example, a composition upregulates the production of OP-1 by a kidney cell line, it would then be desirable to test systemic administration of this compound in an animal model to determine if it upregulates the production of OP-1 *in vivo*. The level of morphogen in the body may be a result of a wide range of physical conditions, *e.g.*, tissue degeneration such as occurs in diseases including arthritis, emphysema, osteoporosis, kidney diseases, lung diseases, cardiomyopathy, and cirrhosis of the liver. The decrease in level of morphogens in the body may also occur as a result of the normal process of aging. The same strategy is used for compositions affecting intracellular pathway components. A composition selected by these screening methods is then used as a treatment or prophylactic.

An appropriate test cell is any cell comprising DNA defining a morphogen-responsive transcription activating element operatively associated with a reporter gene encoding a detectable phenotype-specific gene product. Such DNA can occur naturally in a test cell or can be a transfected DNA. The induced intracellular effect typically is characteristic of morphogenic biological activity, such as Smad activation, or activation of a cascade of biochemical events, such as described above, or involving, for example, cyclic nucleotides, diacylglycerol, and/or and other indicators of intracellular signal transduction such as activation or suppression of gene expression, including induction of mRNA resulting from gene transcription and/or induction of protein synthesis resulting from translation of mRNA transcripts indicative of tissue morphogenesis. Exemplary morphogen-responsive cells are preferably of mammalian origin and include, but are not limited to, osteogenic progenitor cells; calvaria-derived cells; osteoblasts; osteoclasts; osteosarcoma cells and cells of hepatic or neural origin. Any such morphogen responsive cell can be a suitable test cell for assessing whether a candidate substance induced is a morphogen analog.

A preferred identification method is carried out by exposing a test cell to at least one candidate substance, and detecting whether such exposure induces expression of the detectable phenotype-specific gene product that is in operative association with the morphogen-responsive transcription activating element. Expression of this gene product indicates that the candidate substance induces a morphogen-mediated biological effect. Skilled artisans can, in light of guidance provided herein, construct a test cell with a responsive element from a morphogen-

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responsive cell and a reporter gene of choice, using recombinant vectors and transfection techniques well-known in the art. There are numerous well-known reporter genes useful herein. These include, for example, chloramphenicol acetyltransferase (CAT), luciferase, human growth hormone (hGH), beta-galactosidase, and assay systems and reagents which are available through commercial sources. As will be appreciated by skilled artisans, the listed reporter genes represent only a few of the possible reporter genes that can be used herein. Examples of such reporter genes can be found in Ausubel et al., Eds., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, (1989). Broadly, any gene that encodes a detectable product, e.g., any product having detectable enzymatic activity or against which a specific antibody can be raised, can be used as a reporter gene in the present identification method.

A currently preferred reporter gene system is the firefly luciferase reporter system. Gould et al., *Anal. Biochem.*, 7:404-408 (1988), incorporated herein by reference. The luciferase assay is fast and sensitive. In this assay system, a lysate of the test cell is prepared and combined with ATP and the substrate luciferin. The encoded enzyme luciferase catalyzes a rapid, ATP-dependent oxidation of the substrate to generate a light-emitting product. The total light output is measured and is proportional to the amount of luciferase present over a wide range of enzyme concentrations. CAT is another frequently used reporter gene system; a major advantage of this system is that it has been an extensively validated and is widely accepted as a measure of promoter activity. Gorman et al., *Mol. Cell. Biol.*, 2:1044-1051 (1982), incorporated by reference herein. In this system, test cells are transfected with CAT expression vectors and incubated with the candidate substance within 2-3 days of the initial transfection. Thereafter, cell extracts are prepared. The extracts are incubated with acetyl CoA and radioactive chloramphenicol. Following the incubation, acetylated chloramphenicol is separated from nonacetylated form by thin layer chromatography. In this assay, the degree of acetylation reflects the CAT gene activity with the particular promoter.

Another suitable reporter gene system is based on immunologic detection of hGH. This system is also quick and easy to use. Selden et al., *Mol. Cell. Biol.*, 6:3173-3179 (1986), incorporated by reference herein. The hGH system is advantageous in that the expressed hGH polypeptide is assayed in the media, rather than in a cell extract. Thus, this system does not require the destruction of the test cells. It will be appreciated that the principle of this reporter

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gene system is not limited to hGH but rather adapted for use with any polypeptide for which an antibody of acceptable specificity is available or can be prepared.

A small molecule composition may upregulate a morphogen-activated pathway by acting at any one or more point. For example, small molecule potentiation of the pathway may be initiated at the receptor level. Depending on the pathway, the transmembrane receptors may be Type I and/or Type II, or may be comprise variations on either Type I or Type II receptors. For example, OP-1 is capable of activating regulatory pathways comprising at least two variations of both Type I and Type II receptors (ActR-1 and BMPR-1B, and ActRII and BMPR-II, respectively). A small molecule may stimulate the pathway by acting as a ligand and binding to any of the receptors, thereby inducing phosphorylation of Type I receptors and/or Smad molecules. Similarly, a small molecule may activate the regulatory pathway at the level of the serine/threonine kinase domain of the receptors, thereby stimulating phosphorylation of Type I receptors and/or Smad molecules.

As a further alternative, a small molecule may activate the regulatory pathway at the level of Smad complex formation. A small molecule may stimulate the formation of Smad family homodimers, heterodimers, or other homomeric or heteromeric complexes. Furthermore, a small molecule may activate the pathway by interacting with a Smad molecule or Smad complex, thereby altering its tertiary structure.

Alternatively, or in addition, a small molecule may activate the regulatory pathway by facilitating translocation of a Smad molecule or Smad complex or accumulation of the Smad molecule or Smad complex within the nucleus of the cell. By acting as a DNA binding protein or a transcriptional activator, a small molecule may activate the regulatory pathway by increasing transcriptional activity caused by the Smad molecule or Smad complex.

Furthermore, a small molecule can act to stimulate the regulatory pathway by interfering with an inhibitor of the pathway. For example, Smad6 and Smad7, which are structurally different than Smad1, Smad2, Smad3 and Smad5, act as inhibitors of certain types of desirable phenotype-specific protein expression (*e.g.*, by activating TGF- β to induce scar tissue formation). Smad6 forms a stable association with Type I receptors and interferes with the phosphorylation of other Smad proteins, including Smad2 and Smad 1, and their subsequent

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heteromerization with Smad4. Smad7 also forms a stable association with activated Type I receptors and blocks access and phosphorylation of certain Smad molecules, thereby preventing formation of certain Smad heteromeric complexes. Smad7 also inhibits nuclear accumulation of Smad heteromeric complexes. A small molecule may interfere with the inhibitory activity of these Smad proteins by, for example, tightly binding to either one or both proteins and rendering either protein incapable of stable association with Type I receptors, or by competitively binding and stimulating the morphogen-activated transmembrane receptors. Alternatively, a small molecule may activate the inhibitory effects of these Smads in order to inhibit an undesirable effect (*e.g.*, TGF β activity).

Dedifferentiation of a diseased, damaged, or aged soft tissue cells may result from a disturbance in one or more components of a morphogen-activated regulatory pathway. The most appropriate therapy will become evident by screening the intracellular processes in the diseased, damaged, or aged cell. Upon elucidation of the precise nature of the disturbance in the pathway, a small molecule composition can be designed to rectify or bypass the disturbance, thereby allowing normal expression of phenotype-specific gene products to resume. Examples illustrating useful embodiments of the invention follow.

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Examples

Renal Phenotype Restoration Using Morphogens

Morphogens are expressed in the kidney during development. For example, BMP-3 has been shown to be expressed in developing human kidney. Vukicevic et al., *J. Histochem. Cytochem.* 42: 869-875 (1994). Also, OP-1 (BMP-7) has been shown immunohistochemically to be associated with basement membranes in the convoluted tubules of kidneys of human embryos. Vukicevic et al., *Biochem. Biophys. Res. Commun.* 198: 693-700 (1994). In addition, morphogens are expressed in the adult kidney, and high levels of murine OP-1 expression have been observed in adult mouse kidneys. Ozkaynak et al., *Biochem. Biophys. Res. Commun.* 179: 116-123 (1991). Morphogens aid in the preservation of renal phenotype, *inter alia*, by causing expression of phenotype-specific genes. Methods for increasing expression of such genes by activating a morphogen-induced pathway for phenotype-specific gene expression is addressed below.

A rat partial (5/6) nephrectomy or rat remnant kidney model (RRKM) model is employed essentially as described in Vukicevic et al., *J. Bone Mineral Res.* 2: 533 (1987). Male rats (2-3 months old, weighing about 150-200 g) are subjected to unilateral nephrectomy (either left or right kidney). After approximately one week, 2/3 of the remaining kidney is surgically removed. Immediately following surgery, plasma creatinine and BUN levels rise dramatically due to the loss of renal mass and function. Over the next several weeks of this "acute" failure phase, plasma creatinine and BUN levels of surviving animals decline somewhat toward normal values but remain elevated. Renal function then appears to remain relatively constant or stable for a period of variable duration. After this point, the animals enter a period of chronic renal failure in which there is an essentially linear decline in renal function ending in death. As surgical controls, additional rats are subjected to a "sham" operation in which the kidneys are decapsulated but no renal tissue is removed.

Both nephrectomized and sham-operated rats are maintained for approximately 5-6 months after surgery. At that point, surviving nephrectomized animals have entered chronic renal failure.

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Rats are divided into 8 groups with 12 rats in each group. Two groups of nephrectomized rats are used as controls (Nx controls), with one of those groups receiving no treatment at all, while the other receives injections of only the vehicle buffer. In addition, two groups of sham-operated rats are used as controls (sham controls), with one group receiving only the vehicle buffer, while the other receives a small molecule activator of Smad complex formation. Four experimental groups of nephrectomized rats are employed, receiving the small molecule in solution by intraperitoneal injection. Treated and vehicle-only rats receive three injections per week for 4-8 weeks. Total injection volume is approximately 300 μ l. It is expected that no statistically-significant differences are observed between the two control groups or between the two sham control groups.

Compared to the sham group receiving only vehicle, the Nx control receiving only vehicle is expected to demonstrate significantly ($p < 0.01$) elevated serum creatinine at the end of the study, indicating a significant loss of renal function. Although nephrectomized rats treated with Smad complex-inducing small molecules should not show significantly reduced serum creatinine when compared to the Nx control, nephrectomized rats treated with the small molecule should show significant reductions in creatinine values. Similar results should be observed for serum urea levels. All nephrectomized rats are expected to show significantly higher serum urea when compared to the sham-operated rats.

Histological observations are expected to indicate that, in contrast to the vehicle treated Nx control group, OP-1 treated nephrectomized rats exhibit relatively normal glomerular histology. Histomorphometric analysis is expected to indicate that small molecule Nx rats show reduced incidence of glomerular sclerosis and loop collapse, relatively scattered sclerosis and microaneurysms, and more viable glomeruli compared to Nx control rats. It is expected that such effects are due to increased production of one or more phenotype-specific gene products due to small molecule activation of Smad complex formation in the morphogen-induced pathway described above.

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Upregulation of Morphogen-Activated Regulatory Pathway by Small Molecule Interference of Inhibitory Activity of MAP Kinases

Smad1, a mediator of the morphogen-activated phenotype-specific gene regulatory pathway, is also a target of mitogenic growth-factor signaling through epidermal growth factor and hepatocyte growth factor receptor protein tyrosine kinases (RTKs). Kretzschmar et al., *Nature* 389:618-621 (1997). Phosphorylation occurs at specific serines within the region linking the inhibitory and effector domains of Smad1 and is catalyzed by the Erk family of mitogen-activated protein kinases (MAP kinases). In contrast to the morphogen-stimulated phosphorylation of Smad1, which affects carboxy-terminal serines and induces nuclear accumulation of Smad1, Erk-mediated phosphorylation specifically inhibits the nuclear accumulation of Smad1. Smad1 receives opposing regulatory inputs through RTKs and morphogen receptor serine/threonine kinases. Thus, the Erk family of MAP kinases function to inhibit phenotype-specific protein expression which would ordinarily result from the stimulation of morphogen-activated regulatory pathways.

To interfere with the RTK competitive inhibition of morphogen-activated regulatory pathways, a small molecule composition is prepared. The small molecule composition comprises a mutant growth factor protein molecule. The mutant growth factor protein molecule is capable of binding to epidermal growth factor receptors and/or hepatocyte growth factor receptor but incapable of activating the tyrosine kinase. By binding growth factor receptors and restricting tyrosine kinase activity, the linker domain of Smad1 molecules remains unphosphorylated. The carboxy-terminal domain serines of these Smad1 molecules are phosphorylated by the morphogen receptor serine/threonine kinases, thereby permitting the Smad1 molecule to participate in the regulatory pathway, to translocate into the cell nucleus, where it induces transcription of phenotype-specific genes.

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The stimulating effect of the small molecule composition is further enhanced by the addition of a morphogen to activate the morphogen Type I and Type II receptors. Thus, the small molecule composition acts simultaneously to increase phosphorylation of Smad1 and formation of Smad1 heteromeric complexes and to release inhibition of nuclear accumulation of Smad1 heteromeric complexes.

Stimulation of Morphogen-Regulated Transcriptional Activity by Delivery of Naked Smad1 mRNA into Hepatocytes

Smad1 and other Smad proteins stimulate transcriptional activator sequences in association with phenotype-specific genes. This activity is located in the carboxy-terminal domain and is unmasked upon removal of the amino-terminal domain. The transcriptional activity of Smad1 can be stimulated by morphogen-receptor-mediated signals. Overexpression of Smad1 sensitizes cells to endogenous morphogen signals, or exogenous morphogen stimulation and increases transcriptional activity within the cell.

Using essentially the method of Liu et al., *Nature*, 381: 620-623 (1997), the teachings of which are incorporated by reference herein, mRNA encoding at least the carboxy-terminal domain of Smad1 protein is prepared. Following the general methods of U.S. 5,580,859, and Budker et al., *Gene Therapy* 3(7): 593-598 (1997); the teachings of both of which are incorporated herein by reference, naked mRNA encoding full length Smad1 protein and a reporter gene, such as CAT, is placed in a hypertonic solution. The solution is injected intraportally in a mammalian liver having transiently occluded hepatic veins. Expression of the naked mRNA is verified at least 48 hours later using the appropriate reporter gene assay as described above.

Methods of Treatment, Routes of Administration, and Compositions for Treatment

Methods of treating soft tissue having lost normal cellular phenotype comprise the step of administering a composition capable of stimulating one or more aspects of the morphogen-activated expression pathway described above. Administration may be by any compatible route. Thus, as appropriate, administration may be directly to a local environment of a diseased, damaged, or aged tissue. Other contemplated routes of administration include oral or parenteral, including intravenous and intraperitoneal routes of administration. In addition, administration

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may be by periodic injections of a bolus of a composition, or may be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (*e.g.*, an intravenous bag) or internal (*e.g.*, a bioerodable implant, or a colony of implanted, morphogen-producing cells).

5 Therapeutic compositions contemplated by the present invention may be provided to an individual by any suitable means, directly (*e.g.*, locally, as by injection, implantation or topical administration to a tissue locus) or systemically (*e.g.*, parenterally or orally). Where the composition is to be provided parenterally, such as by intravenous, subcutaneous, intramolecular, ophthalmic, intraperitoneal, intramuscular, buccal, rectal, vaginal, intraorbital, intracerebral, 10 intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal or by aerosol administration, the composition may comprise part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the carrier or vehicle is physiologically acceptable so that, in addition to delivery of the desired composition to the patient, it does not otherwise adversely affect the patient's electrolyte and/or volume balance. The fluid medium for the agent thus can comprise normal physiologic saline (*e.g.*, 9.85% aqueous NaCl, 0.15M, pH 15 7-7.4).

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES (Gennaro, A., ed.; Mack Publ., 1990). Formulations of the 20 therapeutic agents of the invention may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity to help maintain the agent at the desired locus. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, tricalcium 25 phosphate, polybutyrate, lactide, and glycolide polymers and lactide/glycolide copolymers, may be useful excipients to control the release of the agent *in vivo*. Other potentially useful parenteral delivery systems for these agents include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, 30 polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for

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administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration.

Suppositories for rectal administration may also be prepared by mixing the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) with a non-irritating excipient such as cocoa butter or other compositions which are solid at room temperature and liquid at body temperatures.

Formulations for topical administration to the skin surface may be prepared by dispersing the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) with a dermatologically acceptable carrier such as a lotion, cream, ointment or soap. Particularly useful are carriers capable of forming a film or layer over the skin to localize application and inhibit removal. For topical administration to internal tissue surfaces, the agent may be dispersed in a liquid tissue adhesive or other substance known to enhance adsorption to a tissue surface. For example, hydroxypropylcellulose or fibrinogen/thrombin solutions may be used to advantage. Alternatively, tissue-coating solutions, such as pectin-containing formulations may be used.

Where the composition is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the blood-brain barrier, the brain capillary wall structure that effectively screens out all but selected categories of substances present in the blood, preventing their passage into the brain. The blood-brain barrier can be bypassed effectively by direct infusion of the molecule capable of releasing morphogen inhibition (alone or in combination with a morphogen) into the brain, or by intranasal administration or inhalation of formulations suitable for uptake and retrograde transport by olfactory neurons.

Modulation of Apoptosis

Modulation of a cellular pathway that controls expression of a phenotype-specific gene has been demonstrated in the *Drosophila melanogaster* model. A member of the BMP subfamily, DPP, plays an important role during *Drosophila* development to establish the patterning of the dorsal ectoderm, regulate gut morphogenesis, and regulate the growth of imaginal discs such as wings and eyes. The *Drosophila* inhibitor of apoptosis 1 (DIAP1) protein

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in *Drosophila* is an interaction protein of a DPP Type I receptor, Thick veins (Tkv). DIAP1 is a homolog of the baculovirus inhibitor of apoptosis (IAP) protein, also an inhibitor of apoptosis. While similar homologs have been reported in virus (OpIAP, VpIAP), mouse (MIHA, mc-IAP-1), and human (XIAP/nIAP, MIHB/c-IAP1/hIAP2, MICH/c-IAP1/hIAP1, NAPI),

5 *Drosophila* was used as a model system herein for exemplification. Results similar to those shown below are expected in humans and other mammals.

IAPs share conserved regions, including two or three baculovirus IAP repeat (BIR) domains in their N-terminal region, and one RING finger domain in their C-terminal region. IAPs are able to prevent cell apoptosis induced by interleukin-1 β converting enzyme (ICE), a

10 capase family protease. The DIAP1 protein associates with Tkv and others (*e.g.*, tumor necrosis factor receptor associated factor (TRAF) 1 and 2) through the C-terminal RING finger domain of DIAP1.

Apoptosis in *Drosophila* is under control of several genetic elements. Included in these are the *doom* gene, which induces apoptosis in insect cells. Doom is localized in the nucleus, as

15 are its binding proteins. Also involved in apoptosis is Reaper, a 65 amino acid polypeptide. The DIAP2 protein prevents Reaper-induced cell death by binding the BIR domain of DIAP2. Reaper is localized in the cytoplasm, and accumulates in perinuclear locations when the IAPs are present.

Plasmid Construction

20 The bait plasmid, pEG-Tkv, encoding the fusion protein of the LexA DNA binding domain and the cytoplasmic region of Tkv was constructed as follows. The cytoplasmic region of Tkv was amplified by polymerase chain reaction (PCR) from the full length clone, Brk25D2 and inserted between the EcoRI and XhoI sites of pEG202. The prey plasmid of Tkv, pJG-Tkv, was constructed by inserting the EcoRI-XhoI fragment into pJG4-5. Tkv mutants were

25 constructed by site-directed mutagenesis using the Chameleon mutagenesis kit (Stratagene). Tkv (Δ JM) lacks the juxtamembrane region (amino acids 205-254) of the wild type Tkv. Tkv (Q253D) and Tkv (K281R) have aspartic acid instead of glutamine 253, and arginine instead of lysine 281, respectively. pcDNA3-HA was made by inserting an annealed oligonucleotide between the XhoI and XbaI sites of pcDNA3 (Invitrogen). pcDNA3-FLAG was described in

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Okadome et al., *J. Biol. Chem.*, 271: 21687-21690 (1996). The whole coding region of Tkv was amplified by PCR. An EcoRI and an XhoI site was added before the starting codon and in place of the stop codon, followed by insertion between the EcoRI and XhoI sites of pcDNA3-HA. The internal EcoRI site of Tkv was removed by site-directed mutagenesis.

5 The yeast expression plasmids of DIAP1 were made by subcloning the EcoRI-XhoI fragment amplified by PCR into pEG202 and pJG4-5. The coding region of DIAP1, without the stop codon, was subcloned between the EcoRI and XhoI sites of pcDNA-FLAG, yielding FLAG-tagged DIAP1. The DIAP2 plasmids were constructed in a similar manner to DIAP1.

Screening and Interaction Assay

10 To search for proteins that interact with Tkv, the interaction trap screen was used, essentially as described by Gyuris et al., *Cell*, 75: 791-803 (1993); Kawabata et al., *J. Biol. Chem.*, 270: 29628-29631 (1995), both incorporated herein by reference. Briefly, a *Drosophila* imaginal disc cDNA library was screened with the cytoplasmic region of Tkv as a bait. The yeast strain, EGY48, was transformed with the reporter, pSH18-34, and pEG-Tkv. The cDNA library was then introduced into EGY48. The transformants were grown on appropriate selection media, and positive clones were selected depending on β -galactosidase activity and leucine prototrophy. Library plasmids were rescued from EGY48, amplified in bacteria, and sequenced. Interaction assays using the interaction trap were done as described before. Kawabata et al., *J. Biol. Chem.*, 270: 29628-29631 (1995).

Cloning of the Full Length DIAP1

20 One of the positive clones contained a partial C-terminal region of DIAP1 (Figure 3). PCR was performed under standard conditions to amplify the missing N-terminal region from a *Drosophila* 4-8 hour embryo cDNA library in the pNB40 vector. The full coding region of DIAP1 was made by ligating the EcoRI-BanII fragment obtained from PCR and the BanII-XhoI
25 fragment obtained from the interaction trap screen.

Protein Interaction in vivo

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 4.5 g/liter glucose. Cells were transiently

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transfected using DMRIE-C (GibcoBRL) with 10 µg of plasmids. After two days, cells were labeled with 22.8 mCi/ml [³⁵S]methionine and cysteine mixture (Amersham) for 5 hours, and lysed in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 1% Triton X-100 containing 1.5% of aprotinin. Cleared lysates were divide into two tubes and incubated with anti-FLAG M2 (Eastman Kodak) or anti-HA 12CA5 (Boehringer Mannheim) monoclonal antibodies. Immune complexes were bound to protein G-Sepharose (Pharmacia) or protein A-Sepharose (Pharmacia). The precipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8.5% or 10% gel) and analyzed by fluorography or with Fuji BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film).

Results

The interaction trap screen was used to search for proteins that interact with Tkv. A *Drosophila* imaginal disc cDNA library was screened with the cytoplasmic region of Tkv as a bait. Of the one hundred and sixty thousand transformants screened, four positive clones were isolated. One clone encoded *Drosophila* FKBP12, a homolog of human FKBP12 which is known as a binding protein for mammalian Type I receptors, including TβR-I. Another clone encoded a partial C-terminal region of DIAP1 (PC1), a homolog of baculovirus IAP. See Figure 3. The remaining two positive clones were not analyzed.

PCR using a *Drosophila* 4-8 hour embryo cDNA library was performed to obtain the missing N-terminal region. The interaction of the full length DIAP1 with Tkv was examined using the interaction trap. As summarized in Figure 4, DIAP1 strongly interacted with the wild type Tkv, although its interaction was slightly weaker than that of the partial clone, PC1. Mutants of Tkv with different signaling activities were also tested for the interaction with DIAP1. One Tkv mutant replacing glutamine 253 with aspartic acid (QD), reported to have a constitutively kinase activity, showed strong interaction with DIAP1 as well as with PC1. Another mutant replacing lysine 281 with arginine (KR), which is expected to lack the kinase activity, showed weak interaction with DIAP1 and PC1. Also tested was a deletion mutant lacking the juxtamembrane region (amino acids 205-254) at the Type II receptor transphosphorylation sites. The mutant, Tkv (ΔJM), did not interact with PC1 or DIAP1. Contrary to Tkv, Saxophone (Sax), another DPP Type I receptor or Punt, a Type II receptor for

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DPP, did not interact with PC1 or DIAP1. Thus, DIAP1 specifically interacted with Tkv in the yeast system.

DIAP2, a second inhibitor of apoptosis in *Drosophila*, was also tested for interactions with Tkv. As depicted in Figure 3, DIAP2 has three BIR domains, while DIAP1 has two BIR domains. DIAP2 did not show interaction with the wild type or mutants of Tkv, Sax, or Punt in the yeast system.

Various truncated forms (TF) of DIAP1 were constructed to identify the interacting region of DIAP1 with Tkv. See Figure 3. The wild type Tkv only interacted with TF4, but not with the other truncated forms. See Figure 5. Since TF4 only has the RING finger domain, the interacting region is mapped to the RING finger domain in DIAP1. Sax and Punt did not interact with TF4.

The interaction between DIAP1 and Tkv *in vivo* were also examined. DIAP1 was epitope-tagged with FLAG at the C-terminus (DIAP1-FLAG). HA-tagged Tkv and/or DIAP1-FLAG were transiently expressed in COS-7 cells. Labeled lysates were immunoprecipitated with anti-HA or anti-FLAG monoclonal antibodies and then subjected to SDS-PAGE. Each antibody specifically recognized Tkv-HA and DIAP1-FLAG, respectively. Anti-FLAG antibody only coprecipitated Tkv when DIAP1 was expressed, demonstrating that DIAP1 interacts with Tkv *in vivo*. Both constitutively active (QD) and kinase-inactive (KR) mutants interacted with DIAP1 as efficiently as the wild type Tkv.

The interaction of DIAP2 with Tkv *in vivo* were also tested. Tkv-HA and/or DIAP2-FLAG were transiently transfected in COS-7 cells. Although the interaction of DIAP2 with Tkv was not detected in the yeast assay (Figure 4), DIAP2 coprecipitated with the wild type Tkv and also with the QD and KR mutants *in vivo*.

Expression plasmids of the BIR domain (BIR-FLAG) and C-terminal region of DIAP1 (PC1-FLAG) were constructed (Figure 6) in order to determine the region of DIAP1 required for the interaction with Tkv *in vivo*. Tkv-HA was coexpressed with BIR-FLAG or PC1-FLAG. Tkv-HA was detected in a stable complex with PC1-FLAG but not with BIR-FLAG. Consistent with the results in the yeast assay, these results indicate that the interaction region of DIAP1 is the C-terminus, which contains the RING finger domain.

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Thus, Tkv induces apoptosis by suppressing DIAP1 function.

Transfection of Mesenchymal Cells with Smad Proteins and Activation of Transfected Cells with BMPs

Biological effects of different Smad Proteins were examined in C2C12 undifferentiated mesenchymal cells using adenovirus-based vector system. Pathway-restricted Smads (R-Smads) activated by BMP receptors, such as Smad1 and Smad5, induced the production of alkaline phosphatase in C2C12 cells, whereas the R-Smads activated by TGF- β /activin pathway (Smad2 and Smad3) did not. Addition of BMP-6 dramatically enhanced the production of alkaline phosphatase induced by Smad1 or 5, which may be due to the nuclear translocation of R-Smads induced by BMP-6. BMP Type I receptors such as ALK-3, ALK-6, and ALK-2, which are known to activate Smad1 and 5, also induced the production of alkaline phosphatase, in these cells. Anti-Smads, *i.e.*, Smad6 and Smad7, inhibited the production of alkaline phosphatase induced by Smads 1 and 5. R-Smads activated by BMP receptors were detected in the cytoplasm in the presence of Smad6 or Smad7. Thus, osteoblast-differentiation induced by BMPs is mainly mediated by R-Smads activated by BMPs, and the effect of R-Smads can be interfered with by anti- Smads.

The experimental procedures included plasmid construction, cell culture and infection of adenovirus, immunoblotting, assays for alkaline phosphatase and osteocalcin production, analysis for chondrogenesis, and subcellular localization of the Smad proteins. All of these procedures are well known in the art.

Results

Differentiation Induction of C2C12 Cells into Osteoblasts by Smad1 and Smad5--C2C12 undifferentiated mesenchymal cells differentiate into osteoblast-like cells by the treatment of BMP-2, BMP-4, and OP-1/BMP-7. The C2C12 cells were transfected with different DNAs by using the adenovirus-based vector, pAxCawt. Transfection efficiency was very high when determined by staining of the cells by LacZ. Plasmids including cDNAs for different Smads were constructed and transfected into C2C12 cells. Expression of FLAG-epitope tagged Smads were analyzed by immunoblotting using anti-FLAG antibody, and production of alkaline

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phosphatase was determined by staining of the cells after 6 days. Smad1, Smad5 and Smad4 are highly expressed in the C2C12 cells.

When the cells were stained for the production of alkaline phosphatase, the cells transfected with Smad5 or Smad1 were positively stained at high m.o.i. In contrast, Smad2 and Smad3, which are activated by TGF- β and activin receptors, did not induce the synthesis of alkaline phosphatase.

Smad4, the common-mediator Smad in mammals, did not induce the alkaline phosphatase production when infected alone; however, co-infection of Smad4 into Smad1/5-infected cells potentiated the effect of BMP-activated Smads. Smad4 weakly induced the production of alkaline phosphatase in the Smad3-infected C2C12 cells, thus, Smad3 weakly, but significantly, activates the transcription of alkaline phosphatase gene in the presence of Smad4.

BMP-6 is structurally most similar to OP-1/BMP-7. Two-hundred ng/ml of BMP-6 efficiently induced the differentiation of C2C12 cells in osteoblasts. When the C2C12 cells were infected with Smad 1 or Smad5, and treated with 200 ng/ml of BMP-6, production of alkaline phosphatase was dramatically enhanced. Smad2, however, did not facilitate the production of alkaline phosphatase.

Differentiation Induction of C2C12 Cells by BMP Type I Receptors- Among the seven different Type I receptors in mammals (ALK-1 through 7), ALK-3 and ALK-6 function as specific BMP Type I receptors. ALK-2 also binds OP-1/BMP-7 and BMP6 and functions as a BMP Type I receptor. Constitutively active forms of HA-tagged ALK plasmids were infected into C2C12 cells and production of alkaline phosphatase was examined. ALK-3(QD) and ALK-6(QD), as well as ALK-2(QD) strongly induced the synthesis of alkaline phosphatase. ALK-1(QD), most similar structurally to ALK-2, also induced the production of alkaline phosphatase. In contrast, ALK-4(TD), ALK-5(TD) and ALK-7(TD) did not induce the alkaline phosphatase activity in the cells, indicating that TGF- β or activin receptors do not efficiently induce the osteoblast differentiation of C2C12 cells.

Nuclear Translocation of Smads Induces Alkaline Phosphatase Synthesis in C2C12 Cells- Subcellular localization of Smad was determined by indirect immunofluorescence staining of the cells using the anti-FLAG antibody to Smad5. Smad5 was observed mainly in the

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cytoplasm. Treatment of the cells with Smad5 and ALK-2(QD) with or without BMP-6 strongly induced the nuclear translocation of Smad4. ALK-3(QD) and ALK-6(QD) also induced the nuclear translocation of Smad5. Overexpression of Smad5 does not result in the nuclear accumulation of Smads, although small amounts of Smad5 may spontaneously translocate into the nucleus. Potentiation of the effect of BMP-6 is thus induced by nuclear translocation of Smad5.

Anti-Smads Block the Differentiation of C2C12 Cells into Osteoblasts Induced by BMPs- Smad6 and Smad7 inhibit the transcriptional activity of R-Smads when assayed using p3TP-lux promoter and cyclin A promoter. It was also shown that Smad6 and Smad7 in mammals as well as *Xenopus* prevent the BMP activity in *Xenopus* embryo assays. However, the effects of anti-Smads on differentiation of osteoblasts have not been examined. C2C12 cells were transfected with Smad6 or Smad7 and treated with BMP-6. Expression of Smad6 and Smad7 correlated with m.o.i. when determined by immunoblotting. Synthesis of alkaline phosphatase was induced by BMP-6, which was not affected by a control plasmid expressing LacZ. Both Smad6 and Smad7 inhibited the production of alkaline phosphatase induced by BMP-6.

When the ALK-3(QD), ALK-6(QD), or ALK-2(QD) were infected into C2C12 cells, alkaline phosphatase was dramatically induced. Co-infection of Smad6 or Smad7 prevented the differentiation of the cells into osteoblasts depending on the expression of proteins.

Both Smad6 and Smad7 were observed throughout the cells, although Smad6 was observed more in the cytoplasm whereas Smad7 was detected more in the nucleus. Smad5 translocated into the nucleus by the stimulation of ALK-3(QD) or ALK6(QD). However, co-infection of Smad6 or Smad7 blocked the nuclear translocation of Smad5, and the protein was observed only in the cytoplasm. When the cells were stained with anti-Smad5 antibody, Smad7 completely blocked nuclear translocation of Smad5, whereas in the Smad6-infected cell, Smad5 was weakly stained in the nucleus, although alkaline phosphatase synthesis was inhibited as in the Smad7-infected cells. Thus, Smad6 and Smad7 prevent the nuclear translocation of Smad5 and thereby inhibit the differentiation of C2C12 cells into osteoblast-like cells.

Additional aspects and embodiments of the invention are apparent to the skilled artisan.

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What is claimed is:

1. A method for restoring cellular phenotype in a cell affected by disease, damage, or age, the method comprising:
 - activating an intracellular pathway that induces expression of a phenotype-specific gene,
 - thereby to restore cellular phenotype.
2. The method of Claim 1, wherein said pathway is a pathway that is activated by specific binding of a morphogen to its transmembrane receptor.
3. The method of Claim 1, wherein said activating step comprises inducing intracellular formation of a Smad complex capable of inducing expression of a phenotype-specific gene.
4. The method of Claim 3, wherein said Smad complex comprises Smad1 and Smad4.
5. The method of Claim 3, wherein said inducing step comprises phosphorylation of a Smad molecule.
6. The method of Claim 1, wherein said activating step comprises exposing a cell having morphogen type-I and morphogen type-II receptors to a small molecule capable of being an agonist of a morphogen type-I or morphogen type-II receptor.
7. The method of Claim 3, further comprising the step of inducing translocation of said Smad complex in to a cell nucleus.
8. The method of Claim 1, wherein the cell is a hepatocyte.
9. The method of Claim 1, wherein the cell is a renal cell.
10. The method of Claim 1, wherein said activating step comprises inducing the expression of a Smad protein.
11. The method of Claim 1, further comprising the step of transfecting the cell with a DNA encoding a Smad protein.
12. The method of Claim 11, wherein said transfecting step is performed by using an adenovirus-based vector.

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1 13. The method of Claim 11, wherein said transfecting step is performed by using a plasmid
2 including said DNA.

1 14. A method restoring cellular phenotype in a cell affected by disease, damage, or age, the
2 method comprising:

3 inhibiting an intracellular pathway that induces expression of a gene that is an
4 inhibitor of normal phenotype,
5 thereby to restore cellular phenotype.

1 15. The method of Claim 14, wherein said gene encodes TGF- β .

1 16. The method of Claim 14, wherein said inhibiting step comprises inducing expression of
2 Smad6.

1 17. The method of Claim 14, wherein said inhibiting step comprises inducing expression of
2 Smad7.

1 18. The method of Claim 1, wherein said activating step comprises administering a
2 morphogen to a patient.

1 19. The method of Claim 18, wherein said morphogen is selected from the group consisting
2 of OP-1, OP-2, OP-3, BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-9,
3 BMP-10, BMP-11, BMP-12, BMP-13, BMP-15, DPP, Vgl, Vgr-1, GDF-1, GDF-2, GDF-
4 3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-10, GDF-11, GDF-12, 60A, NODAL,
5 UNIVEN, SCREW, ADMP, and NEURAL.

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% SEQUENCE SIMILARITY TO HUMAN OP-1 SEVEN-CYSTEINE DOMAIN

SEQUENCE	% SIMILARITY	% NON CONSERVATIVE
hOP-1	100	0
mOP-1	100	0
hOP-2	97	3
mOP-2	97	3
BMP-5	97	3
BMP-6	96	4
Vgr-1(PT)	94	6
OP-3	91	9
60A	90	10
BMP-4	90	10
BMP-2	89	11
dpp	87	13
UNIVIN	87	13
dpp(PT)	86	14
Vg-1	86	14
CDMP-1	85	15
CDMP-3	83	17
GDF-3	83	17
CDMP-2	82	18
DORSALIN	79	21
GDF-1(PT)	78	22
GDF-10	78	22
BMP-3b	78	22
BMP-10	78	23
BMP-3	78	23
SCREW	77	23
ADMP	77	24
TGF- β 2	73	27
GDF-1	73	28
BMP-9	73	28
NODAL	71	29
Inhibin β A	71	29
BMP-15	71	29
TGF- β 3	69	31
Inhibin β B	69	31
Inhibin β C	69	31
TGF- β 5	67	33
TGF- β 1	67	33
GDF-12	67	33
GDF-11	66	34
TGF- β 4	66	34
GDF-9	66	34
GDF-8	64	36
BMP-11	60	40
GDNF	49	51

FIG. 1

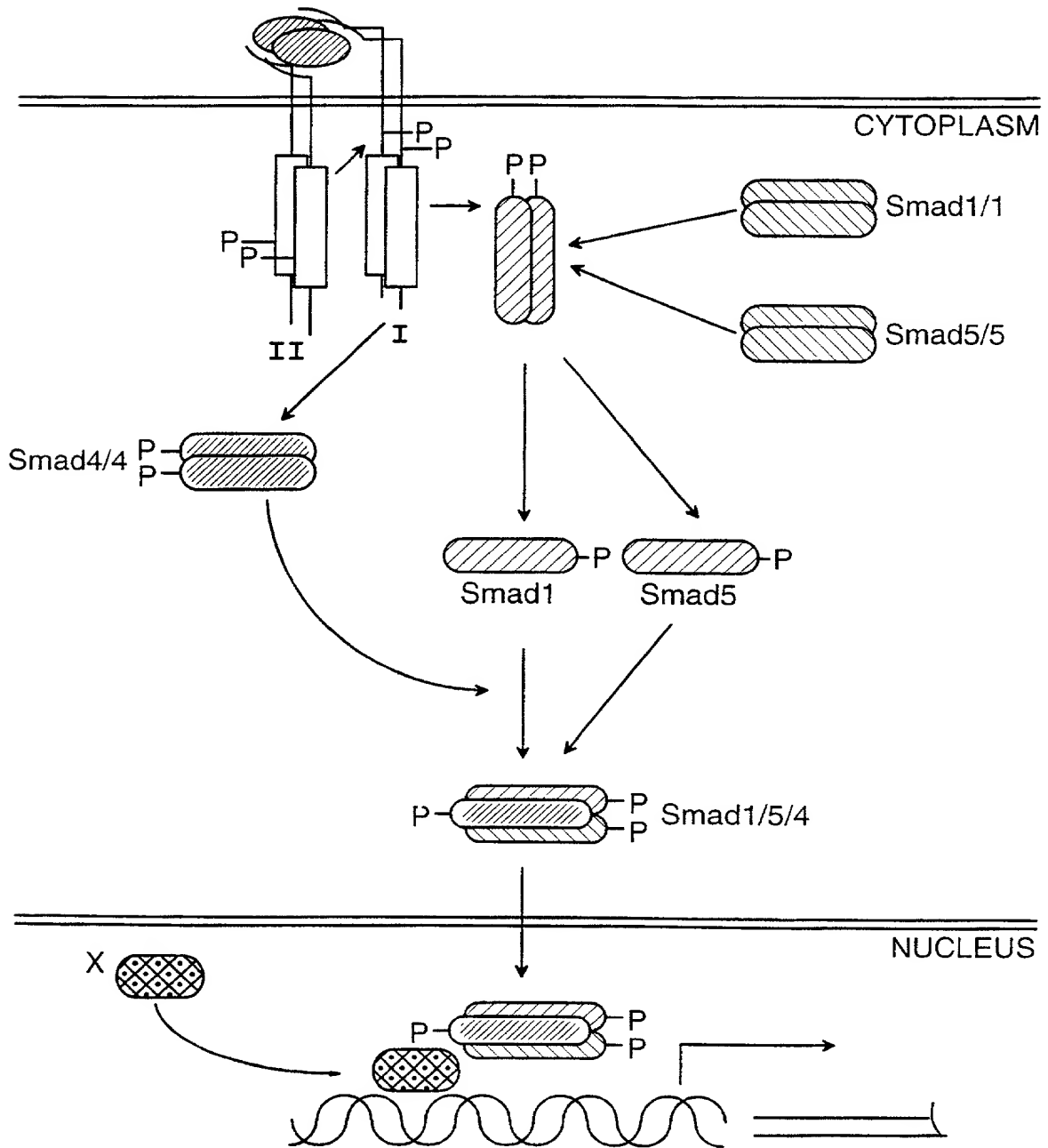
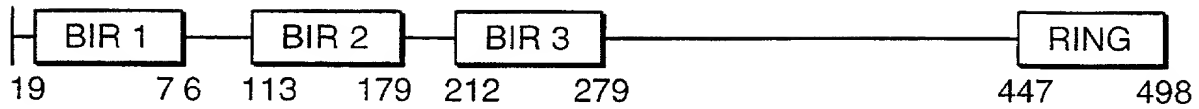


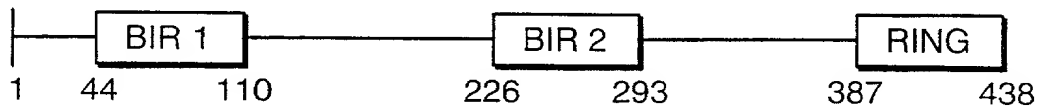
FIG. 2

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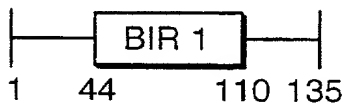
DIAP FULL LENGTH (498 A.A)



DIAP FULL LENGTH (438 A.A)



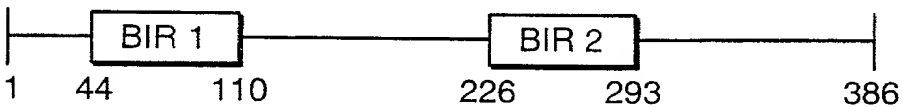
TRUNCATED FORM 1 (TF-1)



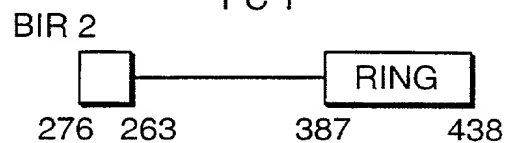
TF 2



TF 3



PC 1



TF 4

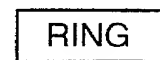


FIG. 3

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	PC1	DIAP1	DIAP2
TKV wt	+++	++	-
TKV QD	++	++	-
TKV KR	+	+	-
TKV Δ JM	-	-	ND
SAX	-	-	-
PUNT	-	-	-

ND : NOT DONE

FIG. 4

	DIAP1	TF 1	TF 2	TF 3	PC1	TF 4
TKV wt	++	-	-	-	+++	++
SAX	-	ND	ND	ND	-	-
PUNT	-	ND	ND	ND	-	-

ND : NOT DONE

FIG. 5

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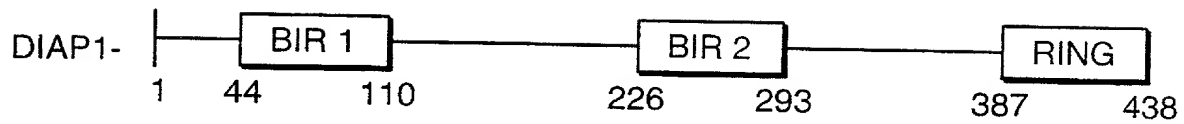


FIG. 6

Date of Deposit:

Attorney Docket No. 00960-520 (CBM-20)

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor which is claimed and for which a utility patent is sought on the invention entitled:

METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE PHENOTYPE OF SOFT TISSUE CELLS

the specification of which:

- ☒ was filed on 12/16/98 as a PCT application designating the United States, and was assigned PCT/US98/26788. A United States national phase application was filed on June 16, 2000 and given U.S.S.N. 09/581,770.
- ☐ is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

- ☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

[illegible]

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Application No. <i>(U.S.S.N.)</i>	Filing Date <i>(dd/mm/yy)</i>	Status <i>(Patented, Pending, Abandoned)</i>
60/069,931	17 December 1997	Abandoned
60/110,498	1 December 1998	Abandoned

PCT International Applications designating the United States:

PCT Appln No.	US Serial No.	PCT Filing Date	Status
WO 99/31136	PCT/US98/26788	12 December 1998	Published

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Brett N. Dorny	35,860	A. Jason Mirabito	28,161
Marianne Downing	42,870	Michel Morency	Limited Recognition
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John A. Harre	37,345	Carol H. Peters	45,010
Shane Hunter	41,858	Thomas M. Sullivan	39,392
David E. Johnson	41,874	Howard Susser	33,556
Kris Kalidindi	41,461	Shelby J. Walker	45,192
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

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- 4 -

[illegible]

☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

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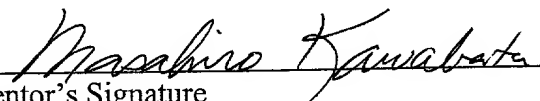
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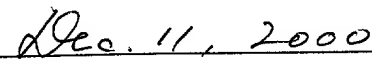
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COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

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METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE PHENOTYPE OF SOFT TISSUE CELLS

the specification of which:

- ☒ was filed on 12/16/98 as a PCT application designating the United States, and was assigned PCT/US98/26788. A United States national phase application was filed on June 16, 2000 and given U.S.S.N. 09/581,770.
- ☐ is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

- ☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

[illegible]

☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Application No. <i>(U.S.S.N.)</i>	Filing Date <i>(dd/mm/yy)</i>	Status <i>(Patented, Pending, Abandoned)</i>
60/069,931	17 December 1997	Abandoned
60/110,498	1 December 1998	Abandoned

PCT International Applications designating the United States:

PCT Appln No.	US Serial No.	PCT Filing Date	Status
WO 99/31136	PCT/US98/26788	12 December 1998	Published

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Attorney or Agent	Registration No.	Attorney or Agent	Registration No.
Kevin N. Ainsworth	39,586	Robert Klauszinski	42,742
Ingrid A. Beattie	42,306	Kristin E. Konzak	44,848
Naomi Biswas	38,384	Cynthia Kozakiewicz	42,764
David F. Crosby	36,400	William A. Marino	44,219
James G. Cullem	43,569	Barry J. Marenberg	40,715
Brett N. Dorny	35,860	A. Jason Mirabito	28,161
Marianne Downing	42,870	Michel Morency	Limited Recognition
Ivor R. Elrifi	39,529	Bradley J. Olson	40,750
Heidi A. Erlacher	45,409	Mike Renaud	44,299
Christina M. Gadiano	37,628	Brian Rosenbloom	41,276
John A. Harre	37,345	Carol H. Peters	45,010
Shane Hunter	41,858	Thomas M. Sullivan	39,392
David E. Johnson	41,874	Howard Susser	33,556
Kris Kalidindi	41,461	Shelby J. Walker	45,192
Christina Karnakis	45,899	Martin M. Zoltick	35,745

all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

Address all telephone calls to Ivor R. Elrifi at telephone number 617/348-1747.

Address all correspondence to:

Ivor R. Elrifi
Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C.
One Financial Center
Boston, Massachusetts 02111

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

Inventor's Signature

Date

Full Name of Inventor: Kuber T. Sampath

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Residence: 98 Pamela Drive, Holliston, MA 01746

Post Office Address: 98 Pamela Drive, Holliston, MA 01746

Inventor's Signature

Date

Full Name of Inventor: Charles M. Cohen

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Post Office Address: 1 Harrington Lane, Weston, MA 02193

Eiichi Oeda

Dec. 12, 2000

Inventor's Signature

Date

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Post Office Address: Kogushi 455-3-B202, Ube, Yamaguchi 755-0067 Japan

Kohei Miyazono

Dec. 10, 2000

Inventor's Signature

Date

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Date

Full Name of Inventor: Masahiro Kawabata

Citizenship: Japan

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Post Office Address: Sakuragaoka 4-23-20, Setagaya-ku, Tokyo 156-0054 Japan

TRADOCS:1340476.1(SQBG011.DOC)

00522210218560

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor which is claimed and for which a utility patent is sought on the invention entitled:

METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE PHENOTYPE OF SOFT TISSUE CELLS

the specification of which:

☒ was filed on 12/16/98 as a PCT application designating the United States, and was assigned PCT/US98/26788. A United States national phase application was filed on June 16, 2000 and given U.S.S.N. 09/581,770.

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Appln. Number	Country (if PCT, so indicate)	Filing Date (dd/mm/yy)	Priority Claimed	
			Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>

00960-520-0000

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Application No. <i>(U.S.S.N.)</i>	Filing Date <i>(dd/mm/yy)</i>	Status <i>(Patented, Pending, Abandoned)</i>
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60/110,498	1 December 1998	Abandoned

PCT International Applications designating the United States:

PCT Appln No.	US Serial No.	PCT Filing Date	Status
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Ingrid A. Beattie	<u>42,306</u>	Kristin E. Konzak	<u>44,848</u>
Naomi Biswas	<u>38,384</u>	Cynthia Kozakiewicz	<u>42,764</u>
David F. Crosby	<u>36,400</u>	William A. Marino	<u>44,219</u>
James G. Cullem	<u>43,569</u>	Barry J. Marenberg	<u>40,715</u>
Brett N. Dorny	<u>35,860</u>	A. Jason Mirabito	<u>28,161</u>
Marianne Downing	<u>42,870</u>	Michel Morency	Limited Recognition
Ivor R. Elrifi	<u>39,529</u>	Bradley J. Olson	<u>40,750</u>
Heidi A. Erlacher	<u>45,409</u>	Mike Renaud	<u>44,299</u>
Christina M. Gadiano	<u>37,628</u>	Brian Rosenbloom	<u>41,276</u>
John A. Harre	<u>37,345</u>	Carol H. Peters	<u>45,010</u>
Shane Hunter	<u>41,858</u>	Thomas M. Sullivan	<u>39,392</u>
David E. Johnson	<u>41,874</u>	Howard Susser	<u>33,556</u>
Kris Kalidindi	<u>41,461</u>	Shelby J. Walker	<u>45,192</u>
Christina Karnakis	<u>45,899</u>	Martin M. Zoltick	<u>35,745</u>

all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

Address all correspondence to:

Ivor R. Elrifi
Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C.
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Boston, Massachusetts 02111

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

Karl T. Sogah

12/22/2020
Date

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Date _____

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4-0
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5-00
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TRADOCS:1340476.1(SQBG01!.DOC)

006227"0/278560

-1-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SAMPATH, K. T.

COHEN, C.

EIICHI, O.

KOHEI, M.

KAWABATA, M.

(ii) TITLE OF INVENTION: METHODS FOR MAINTAINING OR RESTORING TISSUE-APPROPRIATE PHENOTYPE OF SOFT TISSUE CELLS

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: PATENT ADMINISTRATOR, CREATIVE BIOMOLECULES

(B) STREET: 45 SOUTH STREET

(C) CITY: HOPKINTON

(D) STATE: MA

(E) COUNTRY: USA

(F) ZIP: 01748

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: CAMACHO, JENNIFER A.

(B) REGISTRATION NUMBER: P-43,526

(C) REFERENCE/DOCKET NUMBER: CRP-160

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617)-248-7000

(B) TELEFAX: (617)-248-7100

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1067 base pairs

(B) TYPE: nucleic acid

006622 " 022899

-2-

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..1067

(D) OTHER INFORMATION: /product= "MOUSE TYPE 10 COLLAGEN
PROMOTER"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCGATCCTAA AACACTTAAG GATATTTCTG TAAGGCTGTG AAAGAGAAAA CCAACTACTT 60

ACACGGATGG AGACCATGTT TATTTCTTTG GGAGAAAAGC CTAATTGGGA CGCTTCGAGA 120

TCCCTATAGG AAATTGCACC AGTAGTCAAC TGGATTTTAA AAAGGCAAAG CTTGAGGATT 180

TTTTTTTCCC TTTGAAATGA ATGTAGCAAA CTTATGTAAG CACGGAATAG GATTATTAGT 240

TAACAGTCTT TTCAATTATA TGGGAAAATG AAAACTAGGG GAGCGTCTAA GGCCACTTGC 300

TGACCTTTGT GCAGCTGTGA AGTAAAGAAA GTAAACCCTC CAGGGATACT GAACAGCCAA 360

CTGTCATAAG TCCAGGGTGT CTGCACTTG CTGTGACAAG TTTAAAATAT TTAATATGAC 420

TATACCTGAA ATATTTAATG CTATCTTTTT CATGCACCAG CTTCTAAGAG CTTTCCCTAA 480

AATCCTGATA TGCAAAAGAA TATACCAATA TTTTCCCCCT TGCCCTGGC GCTTGTCTCC 540

CAAGTTAGCA AACACTTAGG TAAGCGATTT TTACAGAACT TTTTCCCTA ATAAGTGAAG 600

GACTAACATG ATGATTTAGA TCTATATTCT CCCCAAAGG CGTCTCATAT TTTGTATAT 660

TACCAAATAT TTTCAGTCAA ATAACACAAG AATGTATTTT AAAAATAAAA AGGGTGAATC 720

ATCATTCAT CATGAACCA CATTGGACTC AGAACTCCTA AAAGGAAAAC AGAAAAAAAA 780

AAAAAATCAT GCACAGCCGA AGCTATTAAT ATATAATGGA GACAAAGAGT TTATTTTCA 840

ATGAGAATAA CAAGGAAAAA AGCCTGATTT TGTACGCCTG CCCGTTAGGA CTTCCACCA 900

TAATTAGTGC TTCTTGCCCC TGAGAGGAGG AGCTTCGGCT CAGGGGAACT TCATGCAATA 960

AGGGAAGAAA ACAGTATAAA TACTCCAGGG CAGCCGTGGG GAAGGCATTA TCCACTGCTC 1020

00664770-12900

CTGGGCAGAG GAAGCCAGGA AAGCTGCCCC ACGCATCTCC CAGCACC

1067

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..21
- (D) OTHER INFORMATION: /product= "AP1 SEQUENCE A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCTTGATGA CTCAGCCGGA A

21

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..10
- (D) OTHER INFORMATION: /product= "AP1 SEQUENCE A MUTATION"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTCCTCATCA

10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid

006227 0478660

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..25

(D) OTHER INFORMATION: /note= "Conserved domain of human
c-fos"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Val Glu Gln Leu Ser Pro Glu Glu Glu Lys Arg Arg Ile Arg
1 5 10 15

Arg Ile Arg Asn Lys Met Ala Ala Ala
 20 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..15

(D) OTHER INFORMATION: /product= "AP-1 CONSENSUS SEQUENCE

B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGACTCAGC GCGGA

15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

006227 027350

-5-

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..11
- (D) OTHER INFORMATION: /product= "MEF-2 CONSENSUS"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTAAAAATAA C

11

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1822 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: HOMO SAPIENS
- (F) TISSUE TYPE: HIPPOCAMPUS

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 49..1341
- (C) IDENTIFICATION METHOD: experimental
- (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"
/product= "OP1"
/evidence= EXPERIMENTAL
/standard_name= "OP1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGTGC GGGCC CGGAG CCCCG AGCCCGGGTA GCGCGTAGAG CCGGCGCG ATG CAC GTG
Met His Val

57

1

CGC TCA CTG CGA GCT GCG GCG CCG CAC AGC TTC GTG GCG CTC TGG GCA
Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala

105

00694770 "13390"

5 10 15

CCC CTG TTC CTG CTG CGC TCC GCC CTG GCC GAC TTC AGC CTG GAC AAC 153
 Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn
 20 25 30 35

GAG GTG CAC TCG AGC TTC ATC CAC CGG CGC CTC CGC AGC CAG GAG CGG 201
 Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg
 40 45 50

CGG GAG ATG CAG CGC GAG ATC CTC TCC ATT TTG GGC TTG CCC CAC CGC 249
 Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg
 55 60 65

CCG CGC CCG CAC CTC CAG GGC AAG CAC AAC TCG GCA CCC ATG TTC ATG 297
 Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met
 70 75 80

CTG GAC CTG TAC AAC GCC ATG GCG GTG GAG GAG GGC GGC GGG CCC GGC 345
 Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly Gly Pro Gly
 85 90 95

GGC CAG GGC TTC TCC TAC CCC TAC AAG GCC GTC TTC AGT ACC CAG GGC 393
 Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser Thr Gln Gly
 100 105 110 115

CCC CCT CTG GCC AGC CTG CAA GAT AGC CAT TTC CTC ACC GAC GCC GAC 441
 Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala Asp
 120 125 130

ATG GTC ATG AGC TTC GTC AAC CTC GTG GAA CAT GAC AAG GAA TTC TTC 489
 Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys Glu Phe Phe
 135 140 145

CAC CCA CGC TAC CAC CAT CGA GAG TTC CGG TTT GAT CTT TCC AAG ATC 537
 His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile
 150 155 160

CCA GAA GGG GAA GCT GTC ACG GCA GCC GAA TTC CGG ATC TAC AAG GAC 585
 Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Asp
 165 170 175

TAC ATC CGG GAA CGC TTC GAC AAT GAG ACG TTC CGG ATC AGC GTT TAT 633
 Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile Ser Val Tyr
 180 185 190 195

CAG GTG CTC CAG GAG CAC TTG GGC AGG GAA TCG GAT CTC TTC CTG CTC 681
 Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu Phe Leu Leu
 200 205 210

09581770 "122900

GAC AGC CGT ACC CTC TGG GCC TCG GAG GAG GGC TGG CTG GTG TTT GAC 729
 Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu Val Phe Asp
 215 220 225

ATC ACA GCC ACC AGC AAC CAC TGG GTG GTC AAT CCG CGG CAC AAC CTG 777
 Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg His Asn Leu
 230 235 240

GGC CTG CAG CTC TCG GTG GAG ACG CTG GAT GGG CAG AGC ATC AAC CCC 825
 Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser Ile Asn Pro
 245 250 255

AAG TTG GCG GGC CTG ATT GGG CGG CAC GGG CCC CAG AAC AAG CAG CCC 873
 Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys Gln Pro
 260 265 270 275

TTC ATG GTG GCT TTC TTC AAG GCC ACG GAG GTC CAC TTC CGC AGC ATC 921
 Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe Arg Ser Ile
 280 285 290

CGG TCC ACG GGG AGC AAA CAG CGC AGC CAG AAC CGC TCC AAG ACG CCC 969
 Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro
 295 300 305

AAG AAC CAG GAA GCC CTG CGG ATG GCC AAC GTG GCA GAG AAC AGC AGC 1017
 Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser
 310 315 320

AGC GAC CAG AGG CAG GCC TGT AAG AAG CAC GAG CTG TAT GTC AGC TTC 1065
 Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe
 325 330 335

CGA GAC CTG GGC TGG CAG GAC TGG ATC ATC GCG CCT GAA GGC TAC GCC 1113
 Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala
 340 345 350 355

GCC TAC TAC TGT GAG GGG GAG TGT GCC TTC CCT CTG AAC TCC TAC ATG 1161
 Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met
 360 365 370

AAC GCC ACC AAC CAC GCC ATC GTG CAG ACG CTG GTC CAC TTC ATC AAC 1209
 Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn
 375 380 385

CCG GAA ACG GTG CCC AAG CCC TGC TGT GCG CCC ACG CAG CTC AAT GCC 1257
 Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala
 390 395 400

098170 "12200" 006227

[illegible]